

APPENDIX B

1. A recombinant host cell strain that is the product of a process comprising the steps of:

(a) providing a culture comprised of enteric bacterial host cells comprising a pyruvate formate lyase promoter which is endogenous to said host cells and a DNA encoding a pyruvate formate lyase gene under transcriptional control of said promoter;

(b) transforming host cells in said culture with a heterologous DNA molecule comprising

(i) two genetic elements assembled such that the coding regions of both elements are translated in the same direction, wherein the downstream genetic element comprises a selectable marker gene, a promoter that controls the transcription of said selectable marker gene, and a transcription termination sequence, and wherein the upstream genetic element comprises one or more promoterless coding regions encoding at least one desired polypeptide followed by a transcription termination sequence, and

(ii) sequences that flank said genetic elements and are oriented such that their direction of translation is the same as that of the two heterologous genetic elements, and

(iii) sequences that flank said genetic elements and are sufficiently homologous to said pyruvate formate lyase gene to enable integration by homologous recombination,

whereby integration of said genetic elements into said pyruvate formate lyase gene results by means of homologous recombination;

- (c) selecting for host cells produced in step (b) that express said selectable marker polypeptide at a first level;
- (d) screening host cells obtained in step (c) to obtain host cells that produce said desired polypeptide at an initial level;
- (e) optionally exposing host cells identified in step (d) to a mutagen under conditions such that mutations are created in said DNA; and then
- (f) testing host cells produced in step (d) or step (e) for host cells that produce said marker polypeptide at a level higher than said initial level, to obtain host cells having a mutation that causes increased expression of the upstream genetic element resulting in an increase in production by said host cells of all polypeptides encoded by said heterologous DNA molecule compared to said production of all polypeptides encoded by said heterologous DNA molecule by said host cells in the absence of said mutation, wherein said increased expression is retained in the absence of conditions that select for cells having said increased expression.

2. A cell strain according to claim 1, wherein said strain is a strain of *Escherichia coli* and wherein further

- (i) in step (b) said DNA molecule is a plasmid, wherein said plasmid comprises a replicon that is temperature-sensitive for replication;
- (ii) in step (b) said transforming host cells further comprises introducing said plasmid into said host cells and growing said host cells under conditions that select for cells that express said selectable marker gene at said first level and at a temperature that does not permit replication of said plasmid, resulting in integration of said plasmid into said host gene of said chromosome by homologous recombination; and
- (iii) in step (c) said selecting for host cells further comprises

- (1) growing said host cells that express said selectable marker gene, resulting in excision from said host gene of said temperature-sensitive replicon and of said plasmid, and wherein further
 - (2) said host cells are grown under said conditions at a second temperature that does not permit replication of said plasmid, resulting in host cells that retain said heterologous DNA molecule encoding said desired polypeptide in the absence of said plasmid.
3. A cell strain according to claim 1, wherein further
 - (i) in step (b) said DNA molecule comprises a closed circular DNA lacking an ability to replicate, and
 - (ii) in step (f) said testing host cells comprises selecting for host cells produced in step (d) or step (e) that express said selectable marker polypeptide at a second level that is higher than said first level, and then screening said host cells that express said selectable marker polypeptide at said second level for host cells that produce said desired protein at a level higher than said initial level.
4. A strain according to claim 1, wherein said selectable marker gene confers resistance to chloramphenicol on said host cell strain.
5. A cell strain according to claim 4, wherein said first level of expression of said selectable marker gene confers resistance to at least about 20 µg/ml of chloramphenicol.
7. A cell strain according to claim 4, wherein said coding region of the upstream genetic element of said heterologous DNA segment further comprises a second coding region encoding a second desired polypeptide.

8. A cell strain according to claim 1, wherein said enteric bacterial host cell is selected from the group consisting of *Erwinia chrysanthemi*, *Escherichia coli*, and *Klebsiella pneumoniae*.
9. A cell strain according to claim 8, wherein said enteric bacterial host cell is a cell of a strain of *Escherichia coli*.
10. A cell strain according to claim 7, wherein said coding region of the upstream genetic element of said heterologous DNA molecule encodes an alcohol dehydrogenase and a pyruvate decarboxylase.
11. A cell strain according to claim 10, wherein said alcohol dehydrogenase and said pyruvate decarboxylase are encoded by genes from *Zymomonas mobilis*.
12. A cell strain according to claim 11, wherein said strain is an *Escherichia coli* strain, and said strain is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 90% of added sugar to ethanol.
13. A cell strain according to claim 12, wherein said chromosome further comprises a mutation that impairs succinate production.
14. A cell strain according to claim 13, wherein said mutation that impairs succinate production comprises a mutation in a fumarate reductase (*frd*) gene.
15. A cell strain according to claim 9, wherein said chromosome further comprises a mutation that impairs recombination in said host cell strain.
16. A cell strain according to claim 15, wherein said mutation that impairs recombination comprises a mutation in a *recA* gene.

35. The recombinant host strain, according to claim 1, of *Escherichia coli* KO4 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55123.
36. The recombinant host strain, according to claim 1, of *Escherichia coli* KO11 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55124.
37. The recombinant host strain, according to claim 1, of *Escherichia coli* KO12 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55125.
38. The recombinant host strain, according to claim 1, of *Escherichia coli* KO20 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55126.
39. A cell strain according to claim 11, wherein said cell is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 90% of added sugar to ethanol.
41. A cell strain according to claim 10, wherein said cell strain is designated KO4 and ATCC #55123.
46. A cell strain according to claim 11, wherein said strain is a *Klebsiella oxytoca* strain, and said strain is able to produce ethanol by fermentation of glucose or cellobiose with a theoretical yield corresponding to conversion of at least about 90% of added sugar.
48. (Amended) The recombinant host cell strain according to claim 1, of *Klebsiella oxytoca* M5A1 comprising a plasmid represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 68564.

49. (Amended) A cell strain according to claim 11, wherein said cell strain is designated P2 and represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55307.